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SPONTANEOUSLY ARISING DISEASE

Clinical and Genetic Findings in 28 American Cocker Spaniels with Aural Ceruminous Gland Hyperplasia and Ectasia

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Summary

American Cocker Spaniels (ACSs) develop aural ceruminous gland hyperplasia and ectasia more often than dogs of other breeds. Data on the cause and development of these breed characteristic histopathological changes are lacking. We performed video-otoscopic examinations and dermatological work-up on 28 ACSs, obtained aural biopsies from each dog and assessed the statistical associations between the presence of ceruminous gland hyperplasia and ectasia and disease history, clinical or microbiological findings and underlying cause of otitis externa (OE). Histological lesions of ceruminous gland hyperplasia and ectasia were observed in aural biopsies from 6/13 clinically healthy ears and 13/15 ears with OE from 19/28 examined dogs. Nine of 28 dogs had histologically normal ceruminous glands (odds ratio [OR] 6.2, 95% confidence interval [CI] 1.1–36.6). Bacterial growth in microbiological culture of aural exudate (OR 14.1, 95% CI 2.1–95.3) was associated with ceruminous glandular changes, whereas previous history of OE, cutaneous findings or underlying allergies were not. Pedigree analysis and a genome-wide association study (GWAS) were performed on 18 affected and eight unaffected dogs based on histopathological diagnosis. While the GWAS indicated a tentative, but not statistically significant, association of ceruminous gland hyperplasia and ectasia with chromosome 31, a larger cohort is needed to confirm this preliminary result. Based on our results, ceruminous gland hyperplasia and ectasia may also precede clinical signs of OE in ACSs and a genetic aetiological component is likely Further studies with larger cohorts are warranted to verify our preliminary results.

Keywords: American Cocker Spaniel; chromosome 31; histology; otitis externa

Introduction

Purebred dogs have been developed and maintained to conform to certain aesthetic standards, through selective breeding and the use of inbreeding and popular sires (Björnerfeldt *et al*, 2008; Cruz *et al*, 2008). In addition to predispositions to many disorders due to their conformation, pedigree dog breeds suffer from many inherited diseases (Asher *et al*, 2009). After the dog genome was sequenced in 2005 (Lindblad-Toh *et al*, 2005), over 400 likely causal variants have been reported for canine inherited diseases (www.OMIA.org).

Cocker Spaniels have a breed predisposition to otitis externa (OE) (Angus *et al*, 2002; Saridomichelakis *et al*, 2007; Zur *et al*, 2011). Breed predisposition can be defined as an increased risk for

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a condition in a breed, which may or may not be an inherited disease (Gough *et al*, 2018). The prevalence of OE in American Cocker Spaniels (ACSs) in Finland has been reported to be as high as 27% (Kaimio *et al*, 2017). ACSs are also overrepresented among dogs that require total ear canal ablation and bulla osteotomy (TECABO) surgery as the treatment of choice for end-stage OE (Mason *et al*, 1988; Matthiesen and Scavelli, 1990; White and Pomeroy, 1990; Angus *et al*, 2002; Coleman and Smeak, 2016). In end-stage OE, irreversible pathological changes in the ear canal skin lining contribute to perpetuation of the disease (August, 1988; Harvey *et al*, 2001; Miller *et al*, 2013).

In healthy canine ears, sparsely haired skin covers the inner surface of the ear canal with two types of secretory glands, namely sebaceous and ceruminous glands. The aural epidermis is thin and ceruminous glands are situated below the sebaceous glands in the deeper dermal layers (Nielsen, 1953; Fraser, 1961). Although these glands are present throughout the ear canal, sebaceous glandular tissue increases and ceruminous glandular tissue decreases gradually from the proximal ear canal to its distal portion. However, the density and distribution of the glandular tissue varies markedly between individuals (Huang *et al*, 2009) and breeds. Long-haired breeds have more sebaceous and ceruminous aural glandular tissue than short-haired breeds (Fernando, 1966). Furthermore, the total area of ceruminous glands in the horizontal ear canal is greater in breeds predisposed to otitis, such as Cocker Spaniels (Stout-Graham *et al*, 1990).

In OE, hyperkeratosis and hyperplasia of the epidermis, together with glandular changes, result in narrowing and constriction of the ear canal lumen (Fraser, 1961; Van der Gaag, 1986; Stout-Graham *et al*, 1990). The aural secretory glands become larger and hyperplastic in both the vertical and horizontal ear canals (Huang *et al*, 2009). Breed characteristic histological changes also appear in diseased ears. In Cocker Spaniels, the predominant histological findings of the horizontal ear canal in end-stage OE are ceruminous gland hyperplasia and ectasia, whereas fibrosis is observed in other breeds (Angus *et al*, 2002). The cause of this difference remains unknown.

Aural histology has been studied in dogs using either cadavers or TECABO samples (Fernando, 1966; Van der Gaag, 1986; Stout-Graham *et al*, 1990; Angus *et al*, 2002; Huang *et al*, 2009). There have been few studies on live animals, on early histological findings of the glandular tissue (specifically in ACSs) or of comparisons of aural histological findings with dermatological history and clinical findings. We sought to identify possible associations between breed characteristic ceruminous gland changes and disease history, clinical and microbiological findings of the ears, and the underlying cause of OE. A possible genetic predisposition of ceruminous gland hyperplasia and ectasia in ACSs was also investigated.

Materials and Methods

Study Population

Ethical permissions for this study were granted by the animal ethical committee of the County Administrative Board of Southern Finland (ESAVI-1662/04.10.03/2011 for clinical and histological analysis and ESAVI/7482/04.10.07/2015 for DNA sample collection). The owners of 151 ACSs who had previously answered a questionnaire on ear and skin problems in their dogs (Kaimio *et al*, 2017), ACS breeders and veterinarians treating ACSs were informed of this study. The inclusion criteria for the study were purebred ACSs, with or without a history or presence of ear or skin disease, age ≥12 months and with no contraindications for anaesthesia. The study consisted of one to four visits to the Veterinary Teaching Hospital of the University of Helsinki. The number of visits depended on the diagnostic work-up needed to reveal the primary cause of possible ear or skin disease. The owners of each dog included in the study provided written informed consent for all procedures. The owners were free to withdraw their dogs from the study at any time.

Clinical Evaluation

The same clinician (MK) examined the dogs at each visit. Clinical evaluation included a detailed dermatological history and complete physical, dermatological and video-otoscopic examinations. At the first visit, the ears were examined under sedation, samples for cytology and microbiological culture were collected, and biopsies were taken under anaesthesia. At follow-up visits (which were scheduled if needed), the dermatological and video-otoscopic examinations were repeated awake.

During the first visit, the dogs received medetomidine 10 μg/kg (Domitor; Orion Pharma, Espoo, Finland) and butorphanol 1 mg/kg (Torbugesic; Fort Dodge Animal Health, Overland Park, Kansas, USA) for sedation. Both ears were examined with a video otoscope (Dr. Fritz GmbH, Tuttlingen, Germany). The presence and amount of oedema or swelling, erythema, erosion or ulceration and exudate in the ear canals were scored on a 0–3 scale based on the Otitis Index Score from Nuttal and Bensignor (2014) as follows: none (score 0), mild (1), moderate (2) and marked (3) changes. The total score for

each ear was 0–12. A clinical score ≥4 differentiated ears with OE from clinically healthy ears.

Samples for cytology and microbiological culture were taken from the junction of the vertical and horizontal canals. For cytology, a non-sterile cotton tip applicator was rotated once and then rolled onto a clean glass slide. Cytology slides were prepared and examined under oil immersion (×1,000) as described by Zur *et al* (2011). Bacteriological swabs were taken using sterile transport swabs (M40 Transystem; Copan Diagnostics, Murrieta, California, USA). The swabs were processed and bacterial identification (including susceptibility testing) was performed as previously described (Grönthal *et al*, 2015).

Ear Canal Biopsies

Ear canal biopsies were taken under intravenous anaesthesia (propofol 1 mg/kg [Propovet; Abbott Laboratories Ltd, Maidenhead, UK]). The left ear canal was selected as the biopsy site in all dogs. Hartmann-Herzfeld ear biopsy forceps (3 mm) were inserted into the ear canal until slight resistance was felt, indicating that the forceps were at the junction of the horizontal and vertical ear canals. The forceps were then opened and pushed towards the skin and a small biopsy was pinched. Video-otoscopic examination confirmed the biopsy site after withdrawal of the forceps. The biopsies were formalin fixed, paraffin embedded and routinely processed for histological examination with haematoxylin and eosin (HE) stain.

Diagnostic Tests for Underlying Disease

Complete blood cell counts, serum biochemistry profiles and serum concentrations of T4 and canine TSH provided information on possible underlying systemic disease. Canine atopic dermatitis (CAD) was diagnosed on the basis of a compatible history and clinical signs of the disease, according to Favrot *et al* (2010), after excluding other diseases with similar signs. An 8-week elimination diet, followed by provocation with the original diet, was used to determine adverse food reactions. IgE antibodies to environmental allergens were identified by serology (Allercept; Heska AG, Fribourg, Switzerland). Three skin biopsies, taken with an 8-mm biopsy punch (Kruuse, Langeskov, Denmark) from the ventral chest, behind the scapulae and from the dorsolumbar area, provided additional information on dermatological diseases (eg, cornification disorders) from different parts of the body. Biopsies were formalin fixed, paraffin embedded and routinely processed for histopathological examination with HE stain.

Histological Examination

Aural glandular tissue was evaluated, especially for the following three criteria characteristic of Cocker Spaniels with end-stage OE: ceruminous gland hyperplasia, ceruminous gland ectasia and pigment-laden macrophages (Angus *et al*, 2002). The presence of breed characteristic ceruminous gland changes in the ear canal biopsy was based on the detection of ceruminous gland hyperplasia, ectasia, or both, and such dogs were labelled as 'affected'. Dogs with normal ceruminous glandular tissue served as controls and were labelled as 'unaffected'. In addition, the status of the sebaceous glands and the presence of fibrosis were recorded, and each specimen was given a subjectively ranked score of normal, mild, moderate or marked regarding hyperplasia and hyperkeratosis of the epidermis and inflammation of the dermis.

Skin biopsies were evaluated for the presence of epidermal hyperplasia or hyperkeratosis and infundibular hyperkeratosis, epidermal or dermal inflammation, and adnexal changes. Epidermal and inflammatory changes were subjectively ranked as normal, mild, moderate or marked regarding the severity of these findings. The same pathologist evaluated all biopsies.

Statistical Analysis

The histological findings on the left ear were used for the statistical analysis. The associations between the presence of breed characteristic ceruminous gland changes and the potential associated factors were investigated with logistic regression models. The list of potential associated factors for the response (eg, age, gender and history of OE) (Fig. 3) were predefined by the investigators. Each of the factors was evaluated separately, using the explanatory factor at hand as the sole fixed factor in the model and the presence of ceruminous gland changes as the response. Factors 'age' and 'score of the left ear' were handled as continuous effects and other factors as categorical effects.

There were only nine dogs without ceruminous gland changes in the study. This led to zero frequencies in some of the categories in part of the conducted analyses. This issue was considered in the modelling by applying Firth's (1993) bias adjustment method, which maximizes a penalized likelihood function, instead of the standard maximum likelihood function. Due to the observed low or zero frequencies, multivariate modelling was not sensible and was therefore not performed.

ORs with 95% CIs were calculated to quantify the results. P values <0.05 were considered statistically significant. These statistical analyses were completed

at 4Pharma Ltd using SAS System for Windows, version 9.4 (SAS Institute, Cary, North Carolina, USA).

Genetic Analysis

Pedigree was drawn around the studied dogs using the genealogy software GenoPro 2.5.4.1 (GenoPro, Waterloo, Ontario, Canada) and pedigree trimming was performed with AncesTrim software (Niskanen *et al*, 2017).

EDTA blood samples (1−3 ml) were available from the histologically confirmed affected (n = 18) and unaffected dogs (n = 8). Genomic DNA from leucocytes was extracted by using a semi-automated Chemagen extraction robot (PerkinElmer Chemagen Technologie GmbH, Baeswieler, Germany) according to the manufacturer's instructions. DNA concentrations and purity were measured with a Nanodrop ND-1000 UV/Vis Spectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA) and samples were stored at −20°C (stocks) and +4°C (dilutions).

Genotyping of the DNA samples was performed by GeneSeek Laboratory (Neogen Genomics, Lincoln, New England, USA) using the Illumina CanineHD BeadChip array with 173,662 markers (San Diego, California, USA). After quality control procedures, only single nucleotide polymorphisms (SNPs) that had a >95% genotyping rate and minor allele frequency of >5%, and conformed to Hardy-Weinberg expectations P <0.0001, were included in the analysis, resulting in a total of 95,420 SNPs. No individuals were removed due to a low genotyping rate (<95%).

A genome-wide association study (GWAS) was performed using PLINK 1.9 software (Chang *et al*, 2015) to compare allele frequencies between affected and unaffected dogs. Genomic control adjusted P values were calculated based on the estimation of the inflation factor λ. Multiple testing correction was implemented by using the Bonferroni method, which set the genome-wide significance level to 5.2×10^{-7}. Genotyping data was also analysed using GEMMA 0.98.1 software (Zhou and Stephens, 2012) with standardized relatedness matrix to adjust for population stratification. The CanFam 3.1 assembly was utilized as the dog reference genome.

Results

Study Population and Histological Findings in Ear Canal Biopsies

Of the 38 dogs fulfilling our inclusion criteria, the ear canal biopsy of 10 dogs was too superficial, leaving 28 dogs (seven males and 21 females) in the study. The ages of the dogs ranged from 1 to 11 years (mean 5.7 years). Ceruminous gland hyperplasia, ectasia, or both, appeared in the aural histological samples of 19/28 (68%) dogs (affected), whereas samples from 9/28 (32%) dogs showed normal ceruminous glands (unaffected). Most dogs in both the affected and the unaffected groups had a history of otitis and approximately half had a history of other skin disease (Tables 1 and 2).

At the time of presentation, 13 dogs had clinically healthy ears, whereas 15 dogs had OE, according to our otitis scoring (Tables 1 and 2). Of the 13 dogs with clinically healthy ears, seven had histologically normal ceruminous glands (unaffected), whereas six had ceruminous glandular changes (affected). In the aural histological samples from these six clinically healthy but histologically affected dogs, ceruminous gland hyperplasia and ectasia (n = 2) or focal (n = 1) or multifocal (n = 1, Fig. 1B) ceruminous gland ectasia was seen without signs of inflammation in four biopsies. Two biopsies revealed ceruminous gland hyperplasia and focal ectasia with either a mild diffuse mononuclear cell inflammation in the dermis or mild periadnexal inflammation with pigment-laden macrophages.

From the 15 dogs with OE, 13 had ceruminous gland changes (affected dogs) but two had normal adnexae (unaffected dogs). In the biopsies from the 13 dogs with histologically confirmed OE, ceruminous gland hyperplasia and ectasia occurred together with mainly moderate or marked, diffuse or periadnexal dermal inflammation (Table 1, Fig. 2). The specific inflammatory component consisted of lymphoplasmacytic inflammatory cells (n = 7); most were accompanied with pigment-laden macrophages, neutrophilic inflammatory cells (n = 1) or only periadnexal pigment-laden macrophages (n = 5).

Of the nine unaffected dogs (histologically normal ceruminous glands in the biopsy, Fig. 1A), seven had clinically healthy ears and two had OE. The biopsies from these two dogs showed mild superficial or diffuse lymphoplasmacytic dermal inflammation but normal sebaceous and ceruminous glands (Table 2).

In the statistical analysis, age, gender and disease history had no associations with the presence of ceruminous gland hyperplasia and ectasia. The presence of OE (clinical ear score ≥4, P = 0.04) increased the risk of ceruminous gland changes (OR 6.2, 95% CI 1.06−36.55; Fig. 3). In addition, increase in the clinical score also increased the proportion of dogs with ceruminous gland changes (OR 1.60, 95%CI 1.09−2.34; P = 0.007).

Table 1
Clinical signs and histopathological findings in ear and skin biopsies in American Cocker Spaniels with histologically abnormal ceruminous glands (n = 19)

Patient no.	*Clinical signs*	*Otitis Index Score**	*Dermatological findings*	*Ear canal biopsy findings*	*Skin biopsy findings*	*Underlying cause of ear or skin disease*
22	Only skin problems	0	Mild greasy scaling, mild interdigital erythema	Mild epidermal hyperplasia and hyperkeratosis, focal infundibular hyperkeratosis. Ceruminous gland ectasia, mild diffuse mononuclear cell inflammation	Mild hyperkeratosis, mild perivascular lymphocytic inflammation	CAD
10	Occasional otitis† and skin problems	1	No lesions	Mild epidermal hyperplasia and moderate hyperkeratosis. Ceruminous gland hyperplasia and focal ectasia, pigment-laden macrophages	Normal skin	Unknown
5	Recurrent otitis‡ and skin problems	1	Mild interdigital erythema	Mild epidermal hyperplasia and hyperkeratosis. Ceruminous gland hyperplasia and ectasia	Mild epidermal and infundibular hyperkeratosis, mild superficial eosinophilic inflammation	Allergy, allergens unidentified§
7	No history of ear or skin disease	2	No lesions	Mild epidermal hyperplasia. Ceruminous gland hyperplasia and ectasia with periadnexal pigment-laden macrophages	Normal skin	-
26	Only skin problems	2	Papules, epidermal collarettes and suppurative exudate on the skin of axillae and dorsum, moderate scaling	Mild epidermal hyperplasia and hyperkeratosis, focal infundibular hyperkeratosis. Multifocal ceruminous gland ectasia	Mild epidermal and infundibular hyperkeratosis, focal perifollicular neutrophilic inflammation	CAD
6	Recurrent otitis and skin problems	3	Mild interdigital erythema and scaling	Mild epidermal hyperplasia and hyperkeratosis. Ceruminous gland hyperplasia and ectasia	Mild diffuse hyperkeratosis, mild superficial lymphocytic inflammation	CAD, AFR
35	Occasional otitis	4	Mild interdigital, inguinal and axillary erythema and lichenification	Moderate epidermal hyperplasia and hyperkeratosis, infundibular hyperkeratosis. Ceruminous gland hyperplasia and ectasia, mild diffuse lymphocytic inflammation	Mild epidermal hyperplasia and hyperkeratosis, infundibular hyperkeratosis, mild perivascular lymphocytic inflammation	CAD

(*Continued*)

Table 1 (*continued*)

Patient no.	*Clinical signs*	*Otitis Index Score**	*Dermatological findings*	*Ear canal biopsy findings*	*Skin biopsy findings*	*Underlying cause of ear or skin disease*
21	Occasional otitis and skin problems	4	Perianal superficial pyoderma, mild greasy scaling	Mild epidermal hyperplasia and hyperkeratosis. Focal ceruminous gland ectasia, periadnexal pigment-laden macrophages	Mild epidermal hyperplasia and hyperkeratosis, mild perivascular lymphocytic inflammation	CAD
4	No history of ear or skin disease	6	No lesions	Moderate epidermal hyperplasia and hyperkeratosis. Ceruminous gland hyperplasia and ectasia, moderate diffuse lymphoplasmacytic inflammation with pigment-laden macrophages	Mild epidermal hyperplasia, moderate diffuse and infundibular hyperkeratosis, mild superficial eosinophilic inflammation	CAD
14	Occasional otitis	6	Mild dry scaling	Moderate epidermal hyperplasia and hyperkeratosis. Ceruminous gland hyperplasia and ectasia, mild diffuse lymphoplasmacytic inflammation	Mild epidermal hyperkeratosis, mild superficial perivascular lymphocytic inflammation	Unknown
20	Recurrent otitis	6	Mild interdigital erythema	Moderate epidermal hyperplasia and hyperkeratosis. Ceruminous gland hyperplasia and ectasia, marked diffuse lymphoplasmacytic inflammation	Moderate epidermal hyperkeratosis, infundibular hyperkeratosis	CAD
28	Recurrent otitis	6	Mild inguinal hyperpigmentation and lichenification	Moderate epidermal hyperplasia and hyperkeratosis. Ceruminous gland hyperplasia and ectasia, moderate diffuse plasmacytic and neutrophilic inflammation, pigment-laden macrophages	Normal skin	Unknown
31	Recurrent otitis and skin problems	6	Mild dry scaling	Moderate epidermal hyperplasia and hyperkeratosis. Ceruminous gland hyperplasia and ectasia, marked diffuse inflammation with pigment-laden macrophages	Normal skin	Allergy, allergens unidentified

(*Continued*)

Table 1 (*continued*)

Patient no.	*Clinical signs*	*Otitis Index Score**	*Dermatological findings*	*Ear canal biopsy findings*	*Skin biopsy findings*	*Underlying cause of ear or skin disease*
18	Recurrent otitis and skin problems	6	Moderate inguinal and perianal hyperpigmentation, lichenification and alopecia, moderate dry scaling	Moderate epidermal hyperplasia and mild hyperkeratosis. Ceruminous gland hyperplasia and ectasia with pigment-laden macrophages	Moderate diffuse and infundibular hyperkeratosis	Allergy, allergens unidentified
34	Recurrent otitis	7	No lesions	Mild epidermal hyperplasia and hyperkeratosis, infundibular hyperkeratosis. Ceruminous gland ectasia and moderate diffuse plasmacytic inflammation	Mild diffuse hyperkeratosis, focal perivascular lymphocytic inflammation	Unknown
27	Recurrent otitis	7	Mild interdigital, inguinal and axillary erythema, hyper-pigmentation and lichenification, moderate dry scaling	Marked epidermal hyperplasia and hyperkeratosis. Ceruminous gland ectasia and pigment-laden macrophages	Normal skin	Unknown
2	Recurrent otitis and skin problems	7	Mild interdigital erythema, mild dry scaling	Moderate epidermal hyperplasia and hyperkeratosis, infundibular hyperkeratosis. Ceruminous gland hyperplasia and ectasia, pigment-laden macrophages	Normal skin	Allergy, allergens unidentified
25	Recurrent otitis and skin problems	7	Mild interdigital erythema, inguinal papules, crusts and epidermal collarettes, moderate greasy scaling	Moderate epidermal hyperplasia and hyperkeratosis. Marked ceruminous gland hyperplasia and ectasia, moderate diffuse plasmacytic inflammation with pigment-laden macrophages	Mild epidermal hyperplasia and hyperkeratosis, mild superficial lymphocytic inflammation	AFR
13	Recurrent otitis and skin problems	7	Mild perianal and periocular erythema, lichenification and alopecia, mild dry scaling	Moderate epidermal hyperplasia and hyperkeratosis. Ceruminous gland hyperplasia and ectasia, moderate periadnexal lymphoplasmacytic inflammation with pigment-laden macrophages	Normal skin	CAD

-, clinically healthy dog, no underlying cause of ear or skin disease was investigated; CAD, canine atopic dermatitis; AFR, adverse food reactions.
*Otitis Index Score for left ear. The presence and amount of oedema or swelling, erythema, erosion or ulceration and exudate in the ear canal were scored on a 0–3 scale as follows: none (score 0), mild (1), moderate (2) and marked (3). Total score was 0–12. Clinically healthy ear, total score 0–3; diseased ear, total score ≥4.
†Occasional otitis: a maximum of two otitis episodes per year.
‡Recurrent otitis: at least three otitis episodes per year.
§History, clinical signs and findings compatible with allergy, but an elimination diet trial was not completed as directed or clinically significant level of IgE antibodies not detected.

Table 2
Clinical signs and histological findings in ear and skin biopsies from American Cocker Spaniels with histologically normal ceruminous glands (n = 9)

Patient no.	*History*	*Otitis Index Score**	*Dermatological findings*	*Ear canal biopsy findings*	*Skin biopsy findings*	*Underlying cause of ear or skin disease*
37	No history of ear or skin disease	0	Mild interdigital erythema, lichenification and hyperpigmentation	Normal ear canal	Mild diffuse hyperkeratosis, mild perivascular inflammation with eosinophils, mast cells and lymphocytes	-
16	Occasional otitis†	0	No lesions	Normal ear canal	Mild epidermal and infundibular hyperkeratosis, mild focal perivascular lymphocytic inflammation	Unknown
24	Occasional otitis	0	Mild dry scaling	Normal ear canal	Normal skin	Unknown
12	Recurrent otitis‡ and skin problems	0	Mild interdigital erythema	Normal ear canal	Normal skin	CAD
19	No history of ear or skin disease	1	No lesions	Normal ear canal	Normal skin	-
17	Only skin problems	3	No lesions	Normal ear canal	Mild hyperkeratosis, mild superficial perivascular lymphocytic inflammation	Unknown
3	Recurrent otitis and skin problems	3	Mild interdigital and perianal erythema, mild scaling	Normal ear canal	Normal skin	Allergy, allergens unidentified§
23	Recurrent otitis and skin problems	4	Perianal hyperpigmentation, lichenification and alopecia	Mild epidermal hyperplasia and moderate hyperkeratosis. Mild diffuse plasmacytic inflammation. Normal adnexae	Normal skin	CAD, AFR
36	Occasional otitis	5	Mild dry scaling	Marked epidermal hyperplasia and moderate hyperkeratosis. Superficial neutrophilic and plasmacytic inflammation. Normal adnexae	Mild epidermal hyperplasia, moderate diffuse and infundibular hyperkeratosis, mild perivascular lymphocytic inflammation	Unknown

-, clinically healthy dog, no underlying cause of ear or skin disease was investigated; CAD, canine atopic dermatitis; AFR, adverse food reactions.
*Otitis Index Score of left ear. The presence and amount of oedema or swelling, erythema, erosion or ulceration and exudate in ear canal were scored on a 0–3 scale as follows: none (score 0), mild (1), moderate (2) and marked (3). Total score was 0–12. Clinically healthy ear, total score 0–3; diseased ear, total score ≥4.
†Occasional otitis: a maximum of two otitis episodes per year.
‡Recurrent otitis: at least three otitis episodes per year.
§History and clinical findings compatible with allergy, but elimination diet trial was not completed as directed and clinically significant level of serum IgE antibodies not detected.

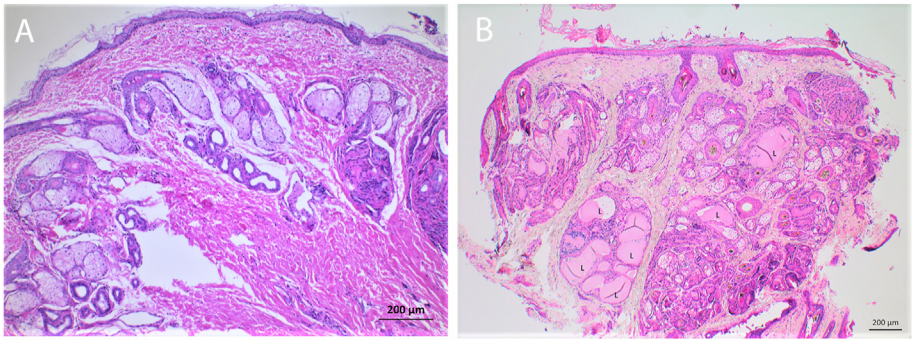

Fig. 1. Dog, ears. (A) Normal ceruminous glands in clinically healthy dog (no. 16). Biopsy. HE. (B) Mild epidermal hyperplasia and compact orthokeratotic hyperkeratosis, mild infundibular hyperkeratosis, numerous sebaceous glands and multifocal ceruminous gland ectasia (L) in ear of affected dog (no. 26). Biopsy. HE.

Fig. 2. Dog, ear, otitis externa. Moderate epidermal hyperplasia and mild compact orthokeratotic hyperkeratosis, marked multifocal ceruminous gland hyperplasia and ectasia, and periadnexal pigment-laden macrophages (dog no. 18). HE.

Microbiological Findings in Aural Exudate

Malassezia spp yeasts were present in the cytological samples from both the affected and unaffected groups (11/19 [58%] and 4/9 [44%], respectively), whereas cocci (9/19; 68%) and rods (8/19; 42%) were found only in samples from the affected dogs. In microbiological cultures, bacterial growth was detected in the samples of 16/19 (84%) affected and 2/9 (22%) unaffected dogs. Mixed bacterial growth appeared in 10/19 (53%) samples from affected dogs. Altogether, 18 different species were identified. *Staphylococcus pseudintermedius* (n = 11) was the most prevalent bacterial species detected, followed by *Corynebacterium auriscanis* (n = 5) and *Streptococcus canis* (n = 5). Methicillin-resistant *S. pseudintermedius* was present in two samples as well as *Pseudomonas aeruginosa* and *Escherichia coli*. The presence of bacterial growth (OR 14.1, 95%CI 2.1–93.2; $P = 0.007$) in culture was associated with the presence of ceruminous gland changes.

Dermatological Findings and Underlying Diseases

On dermatological examination, 11/19 (58%) affected and 4/9 (44%) unaffected dogs had signs of inflammation such as erythema, especially in the interdigital skin. In addition, 11/19 (58%) affected and 3/9 (33%) unaffected dogs demonstrated scaling of the skin. Allergies were diagnosed in 13/19 (68%) affected and 3/9 (33%) unaffected dogs. Allergens were identified in 11 dogs; adverse food reactions to beef and wheat as the sole cause of clinical signs in one dog and sensitivities to mites (house-dust mite or storage mites, n = 5), mites and pollens (n = 4) or pollens (n = 1) in 10 dogs with CAD. Two of the dogs with CAD also had adverse food reactions. Five other dogs had a history and clinical findings compatible with allergies, but the allergens were unidentified because the elimination diet trial was not completed as directed or no relevant antibodies were identified by serology. Histological findings in the skin biopsies were non-specific. Dermatological findings are presented in detail in Tables 1 and 2. No associations with ceruminous gland changes were found.

Potential Genetic Contribution to Aetiology

To study the possible genetic component of disease aetiology, a pedigree analysis and GWAS were conducted. A pedigree drawn around the histologically confirmed affected (n = 18) and unaffected dogs (n = 8) indicated that many of the affected dogs were closely related and there were multiple affected dogs in some of the affected litters, suggesting a strong genetic contribution (Fig. 4).

To map the disease locus, a GWAS was performed with the available affected and unaffected dogs. After quality control, 95,420 markers were included in the analysis. Analysing the data with Plink indicated no statistically significant association. However, the strongest potential association was observed in

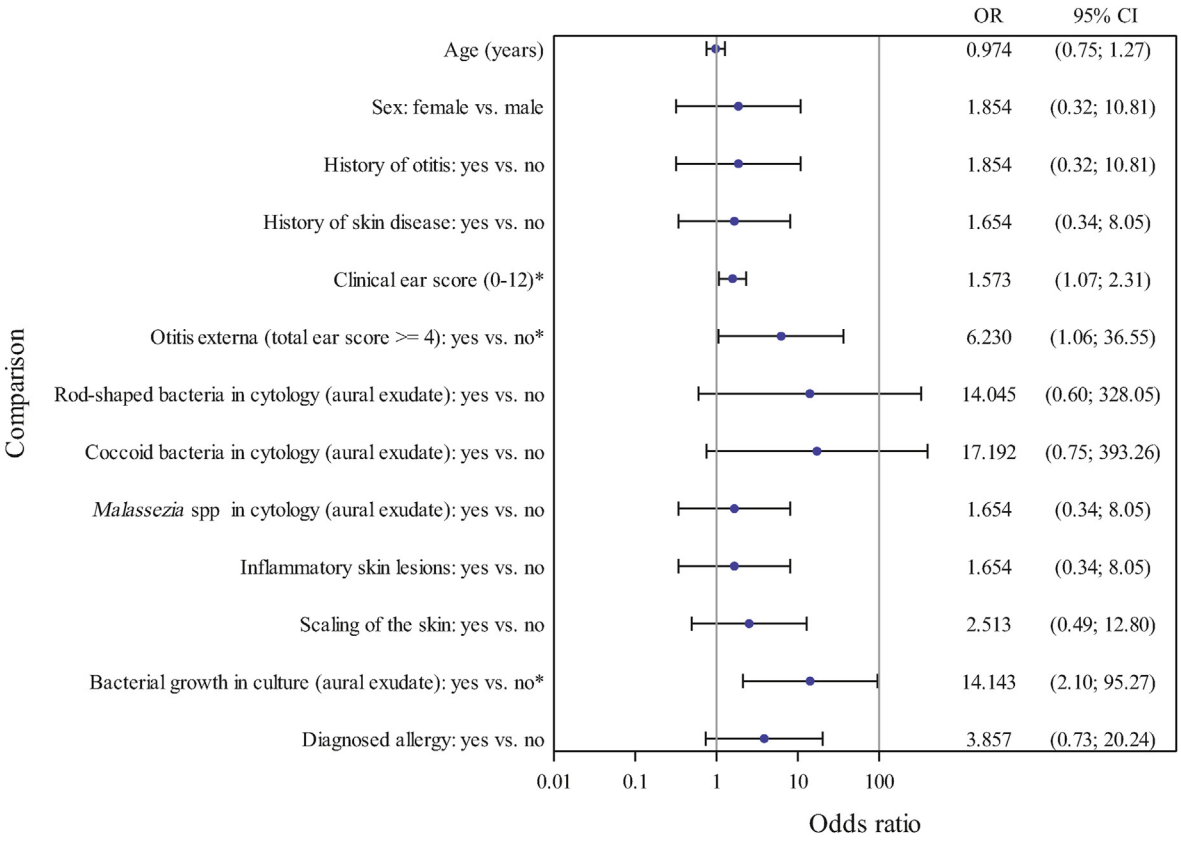

Fig. 3. Associations between potential explanatory factors (y-axis) and the presence of ceruminous gland hyperplasia and ectasia were investigated with univariate logistic regression analyses. X-axis shows the odds ratio for effect of explanatory factor on risk of dog having ceruminous gland hyperplasia and ectasia. Blue dot in horizontal bar represents the calculated odds ratio and the bar represents the confidence interval. Statistically significant associations (asterisks). CI, confidence interval; OR, odds ratio.

chromosome 31 (CFA31) with top SNPs BICF2P1008320 at 13,969,467 bp and BICF2S23158068 at 14,059,798 bp ($P_{GC} = 4.0 \times 10^{-5}$, $\lambda = 1.22$, Figs. 5A, B and G), respectively. The assessment of the genotypes in this region revealed that 13/18 affected dogs, but also one of the unaffected dogs, shared a homozygous haplotype of 115.6 kb spanning from 13,944,155 bp to 14,059,798 bp (Fig. 5C). The region and its close proximity (+/− 1 Mb) includes 21 genes and gene predictions in Ensembl, including functionally potential candidate genes CXADR Ig-like cell adhesion molecule (*CXADR*), BTG anti-proliferation factor 3 (*BTG3*) and transmembrane serine protease 15 (*TMPRSS15*).

The GWAS analysis was repeated with GEMMA, which better adjusts for population structure. This analysis also indicated a region in CFA31 with top SNP being BICF2P84320 at 16,317,450 bp ($P = 1.4 \times 10^{-6}$, $\lambda = 1.12$, Figs. 5D, E and H); the following two were the same as the top markers in the previous analysis using Plink (BICF2P1008320 at 13,969,467 bp and BICF2S23158068 at 14,059,798 bp; $P = 9.0 \times 10^{-6}$). Importantly, none of the markers met the genome-wide significance level, indicating a larger sample cohort is needed. Assessment of the genotypes in the tentative locus showed that 17/18 affected dogs, but again also one of the unaffected dogs, shared a homozygous haplotype block of 98.4 kb spanning from 16,235,099 bp to 16,333,473 bp (Fig. 5F). This region and its close proximity (+/− 1 Mb) contains 11 genes and gene predictions in Ensembl, including the protein-coding gene neural cell adhesion molecule 2 (*NCAM2*).

Discussion

In this study, we compared aural histology with disease history and clinical findings, and performed genetic analysis for the studied dogs based on histopathological diagnosis. Ceruminous gland hyperplasia, ectasia, or both, occurred in 68% of the studied dogs, in both dogs with clinically healthy ears and dogs with OE. Ceruminous gland hyperplasia and ectasia were associated with OE, and marked

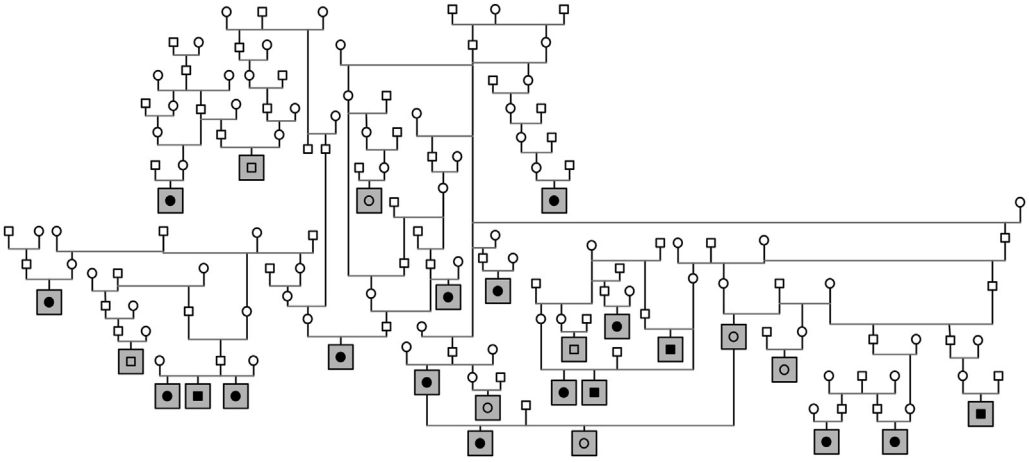

Fig. 4. Pedigree analysis suggests hereditary component of ceruminous gland hyperplasia and ectasia in American Cocker Spaniels, but mode of inheritance could not be assessed due to the limited number of reliably phenotyped dogs. Circles and squares indicate females and males, respectively. Histologically confirmed affected dogs (n = 18) marked with black and unaffected dogs (n = 8) with grey; undetermined dogs are white. Genotyped dogs (n = 26) marked with grey rectangle.

histological changes appeared in affected ears. In ears with OE, ceruminous gland changes seemed extremely common in this breed, as 13/15 (87%) dogs had such findings. This is consistent with a previous study, in which only 6% of Cocker Spaniels with end-stage OE had normal ceruminous glands (Angus *et al*, 2002).

Interestingly, ceruminous gland hyperplasia and ectasia also appeared in six dogs with clinically healthy ears. Three of these dogs had a history of OE, thus ceruminous gland hyperplasia and ectasia may have been caused by a previous OE episode. To the authors' knowledge, however, the time period needed for the aural glandular tissue to normalize after inflammation is unknown. In addition, in our study a history of previous OE showed no association with ceruminous gland changes. Furthermore, three clinically healthy dogs with no history of OE also had ceruminous gland hyperplasia and ectasia. This novel finding suggests that in ACSs, ceruminous gland ectasia and hyperplasia may also precede clinical signs of OE. Cocker Spaniels have been proposed to have a breed-related, profound and predominantly ceruminous tissue response to inflammatory stimuli of the ear canal (Angus *et al*, 2002). It is possible that, due to a genetic predisposition, the aural glandular tissue responds to minimal inflammatory stimuli. Another possibility is that the aural glandular tissue might be primarily overactive in some dogs, as an evident cause for the observed ceruminous gland hyperplasia and ectasia was not found. Of the various primary causes of otitis, glandular disorders are poorly documented in the dog and cat (Miller *et al*, 2013). As the sebaceous glands secrete most of the skin surface lipids (Lloyd and Garthwaite, 1982; Miller *et al*, 2013), ceruminous gland hyperplasia and ectasia may result in a lowering of the lipid content of cerumen (Huang *et al*, 1994). This may cause accumulation of cerumen and possibly maceration of the ear canal skin lining, facilitating secondary infection. The resulting inflammation may further promote histological changes of the ear canal and eventually cause a vicious cycle (Harvey *et al*, 2001).

Interestingly, as in ACSs, ceruminous glandular hyperplasia and ectasia and ceruminous glandular tumours are very common in endangered Santa Catalina Island foxes (*Urocyon littoralis catalinae*) in California, USA. These animals are a unique small fox subspecies with a suspected genetic predisposition to tumour development, as foxes in nearby islands do not suffer from these ear conditions. A progressive and persistent severe inflammatory process in response to ear mite infection is suspected to lead to tumour development (Vickers *et al*, 2015). This theory is supported by the finding that acaricide treatment led to a marked decrease in mite burden, inflammation and glandular hyperplasia (Moriarty *et al*, 2015). In addition to ear mite infection, the affected foxes also had decreased microbial diversity in their ear canals, with *S. pseudintermedius* predominant. Thus, it has been suggested that in these foxes, secondary bacterial infection may also contribute to sustained inflammation, ceruminous glandular hyperplasia and tumour development (DeCandia *et al*, 2020). In our study, the presence of ceruminous gland hyperplasia and ectasia was associated with bacterial growth in the ear canal and *S. pseudintermedius* was the most prevalent species isolated. *S. pseudintermedius* is an opportunistic pathogen that resides in dog skin and mucous membranes and primarily causes ear and skin infections

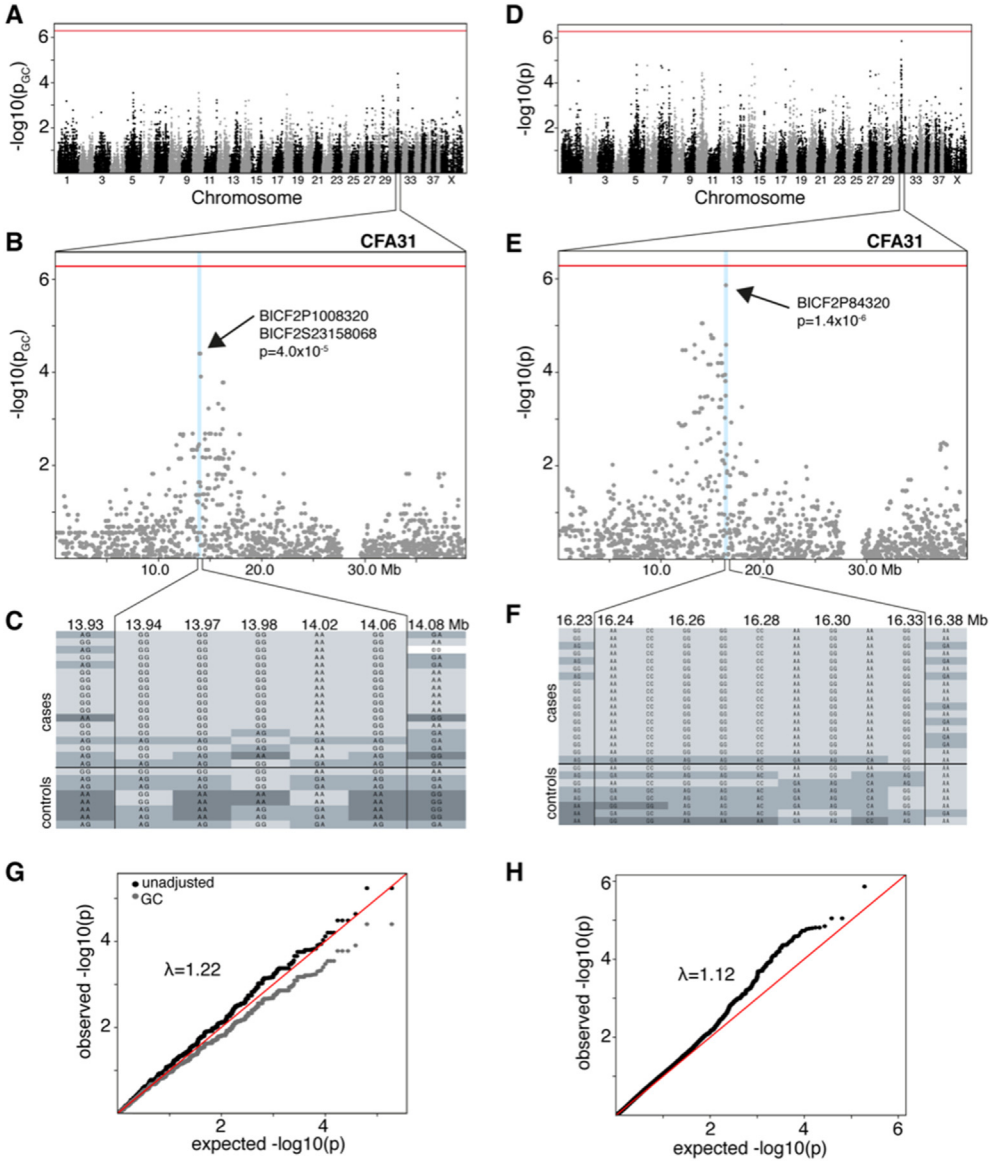

Fig. 5. (A, B) GWAS performed with Plink indicated a tentative association on the CFA31 with top SNPs BICF2P1008320 at 13,969,467 bp and BICF2S23158068 at 14,059,798 bp ($P_{GC} = 4.0 \times 10^{-5}$, $\lambda = 1.22$). Red line indicates the genome-wide significance threshold at 5.2×10^{-7}, which none of the analysed markers met, indicating further studies are needed to confirm the association. (C) A shared homozygous haplotype of 115.6 kb spanning from 13,944,155 bp to 14,059,798 bp was seen in 13/18 affected and 1/8 unaffected dogs. Rows represent individual animals and columns genotypes at each SNP, with light grey denoting homozygotes, intermediate heterozygotes and dark grey opposite homozygotes. (D, E) GWAS was repeated with GEMMA to better control for population structure. This analysis also indicated a tentative association on the CFA31, but with top SNP located at 16,317,450 bp ($P = 1.4 \times 10^{-6}$, $\lambda = 1.12$), while the two following SNPs were the same as the top markers in the Plink analysis ($P = 9.0 \times 10^{-6}$). (F) A shared homozygous haplotype of 98.4 kb spanning from 16,235,099 bp to 16,333,473 bp was seen in 17/18 affected and 1/8 unaffected dogs. The QQ-plots of the GWAS analysis performed with (G) Plink and (H) GEMMA indicate mild to moderate stratification in the cohort that is commonly seen in purebred dogs. A and D show the genome-wide view of the results with chromosomes 1–39 on the x-axis and *P* values on the y-axis. B and E show the association results from chromosome 31 (CFA31). Each dot represents individual markers in the analysis.

when host resistance is lowered or barrier function is altered (Bannoehr and Guardabassi, 2012). In atopic dogs, antimicrobial therapy resulted in improved barrier function and increased microbial diversity (Bradley *et al*, 2016), indicating that host–pathogen interactions may play an important role in the pathogenesis of this inflammatory disease. The possible role of microbes in the pathogenesis of ceruminous gland hyperplasia and ectasia is interesting and warrants further studies.

Previous studies have shown allergies to be the most common primary cause of otitis (Saridomichelakis

et al, 2007; Zur *et al*, 2011). Furthermore, adverse food reactions (Rosser, 1993; Chesney, 2002) and CAD (Hillier and Griffin, 2001) may be manifested solely by OE, or otitis may precede other signs in allergic dermatitis (Picco *et al*, 2008). In the present study, allergies were diagnosed in 65% of the dogs with OE. However, the presence of ceruminous gland changes in a biopsy was not associated with allergies or with signalment, history of ear or skin disease or clinical or histological findings of skin disease. Our preliminary results suggest that ceruminous gland hyperplasia and ectasia are independent of the history, presence of dermatological disease or possible allergic causes of OE. Based on our observations, ceruminous gland hyperplasia and ectasia may be a phenotype fixed in the breed, or most of the dogs may be predisposed to the condition.

Genetic or gene regulatory differences may play a role in the uniquely strong inflammatory response in Santa Catalina Island foxes (Vickers *et al*, 2015; DeCandia *et al*, 2020), and the same might apply to ACSs. We evaluated the possible heredity of ceruminous gland hyperplasia and ectasia from multigeneration pedigrees and GWAS. Many affected dogs were closely related to each other and multiple affected dogs appeared in some of the affected litters. Together with the prevalent breed characteristic ceruminous gland changes (Angus *et al*, 2002), these types of pedigrees suggest a genetic background, although the exact mode of inheritance is challenging to establish due to missing confirmed phenotype information, especially from unaffected dogs. Our GWAS with a small cohort of affected and unaffected dogs suggested a tentative locus in chromosome 31, which needs confirmation in a larger cohort prior to further conclusions. However, in the tentative loci, 13 to 17 out of 18 affected dogs partly shared a homozygous haplotype block, which was also found in one of the unaffected dogs. This particular dog was clinically and histologically healthy at the time of presentation at 1 year of age. However, according to the owner, the dog had required regular ear cleaning due to excessive earwax formation, possibly indicating increased glandular activity of the ear canal skin.

Several protein-coding genes, including *CXADR* and *BTG3*, and long non-coding RNAs with potentially relevant functions in controlling inflammation and regulating cell proliferation are located in the tentative loci and warrant some discussion on their potential role in pathogenesis. CXADR is a transmembrane receptor with a key role in controlling adhesion between adjacent epithelial cells (Schreiber *et al*, 2014; Ortiz-Zapater *et al*, 2017). Differences in CXADR expression have been observed in different malignancies, including skin cancer cell lines and breast cancer (Schreiber *et al*, 2014; Ortiz-Zapater *et al*, 2017; Nilchian *et al*, 2018). Furthermore, interaction of CXADR with junctional adhesion molecule-like protein (JAML) is important for leucocyte migration and for epithelial γδ T cell activation, indicating that CXADR contributes to the control of local immune responses in the skin (Zen *et al*, 2005; Verdino *et al*, 2010; Whitherden *et al*, 2010). CXADR may have an important role in host–pathogen interactions in bovine mammary gland infection (Han, 2019). BTG3 inhibits cell proliferation, metastasis and angiogenesis and regulates cell-cycle progression and differentiation in a variety of cell types. Decreased BTG3 expression is linked to carcinogenesis (Deng *et al*, 2013; Zheng *et al*, 2017). The findings from the GWAS suggest that there may be a genetic factor predisposing ACSs to the condition, but this finding needs replication in additional well-phenotyped samples prior to further conclusions.

The results of this study must be considered within its limitations. Owners of dogs with ear disease might have been more willing to participate in this study, although we also attempted to recruit clinically healthy dogs in the study. Disease history was based on the owner's perception, which can be susceptible to recall bias. The group of studied dogs was small. Although the technique of taking the biopsy with biopsy forceps enabled rapid and safe sampling, some of the biopsies were too superficial and reduced our group of studied dogs. As ear canal biopsy is an invasive procedure requiring anaesthesia, repeated sampling was not considered ethical. The representativeness of local biopsies may be limited and biopsies show only the current status of the dog, which may vary over time. Ceruminous hyperplasia and ectasia are likely a multifactorial disorder in which environmental factors (such as treatment) might affect the phenotype, as is the case in Santa Catalina Island foxes (Moriarty *et al*, 2015). Thus, a larger, well-phenotyped cohort is warranted for further analysis to verify our preliminary results.

In conclusion, ceruminous gland hyperplasia and ectasia in ACSs occurred both in dogs with OE and in dogs with clinically healthy ears. Ceruminous gland changes were associated with OE and there were severe changes in affected ears. However, as clinically healthy ears also had ceruminous gland hyperplasia and ectasia, these changes may precede clinical signs of OE. Our results also suggest that the presence of ceruminous gland changes is not associated with the presence of dermatological disease or allergies, and that there likely exists a genetic background for the condition in this breed. Further studies with a larger number of dogs are needed to confirm these

preliminary findings and to elucidate the cause of ceruminous glandular changes in ACSs.

CRediT Author Statement

Mirja Kaimio: Conceptualization, Methodology, Investigation, Data Curation, Writing - original draft. Sanna Malkamäki: Methodology, Investigation, Writing - review and editing, Visualization. Maria Kaukonen: Methodology, Formal Analysis, Investigation, Writing - original draft, Visualization. Saija Ahonen: Formal Analysis; Writing - review and editing. Marjo K. Hytönen: Conceptualization, Data Curation, Investigation, Supervision, Writing - review & editing. Merja Rantala: Methodology, Investigation, Supervision, Writing - review and editing. Hannes Lohi: Conceptualization, Funding Acquisition, Investigation, Resources, Supervision, Writing - review editing. Leena Saijonmaa-Koulumies: Conceptualization, Methodology, Supervision, Writing - review and editing. Outi Vapaavuori: Conceptualization, Funding Acquisition, Supervision, Methodology, Writing - review and editing.

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Conflict of Interest Statement

The authors declared no conflicts of interest with respect to the research, authorship or publication of this manuscript.

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